DTIC FILE COPY

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS NONE
2a. S 2b. D 4. PE **AD-A217 911**	3. DISTRIBUTION/AVAILABILITY OF REPORT APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED. (1)
	5. MONITORING ORGANIZATION REPORT NUMBER(S) AFIT/CI/CIA- 89-151
6a. NAME OF PERFORMING ORGANIZATION AFIT STUDENT AT UNIV OF FLORIDA 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION AFIT/CIA
6c. ADDRESS (City, State, and ZIP Code)	7b. ADDRESS (City, State, and ZIP Code) Wright-Patterson AFB OH 45433-6583
8a. NAME OF FUNDING/SPONSORING ORGANIZATION 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification) (UNCLASSIFIED)
THE BIOCHEMICAL CHARACTERIZATION OF PLASMA CLASS I HUMAN LEUKOCYTE ANTIGENS (HLA)

12. PERSONAL AUTHOR(S)
JO ANN HAGA

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
THESIS/DISSERTATION	FROM _____ TO _____	1989	58

16. SUPPLEMENTARY NOTATION
APPROVED FOR PUBLIC RELEASE IAW AFR 190-1
ERNEST A. HAYGOOD, 1st Lt, USAF
Executive Officer, Civilian Institution Programs

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

DTIC ELECTE FEB 13 1990 S D

Co D

90 02 12 029

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL ERNEST A. HAYGOOD, 1st Lt, USAF	22b. TELEPHONE (Include Area Code) (513) 255-2259 22c. OFFICE SYMBOL AFIT/CI

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

AFIT/CI "OVERPRINT"

TITLE: The Biochemical Characterization of Plasma Class I Human Leukocyte Antigens (HLA)

AUTHOR: Jo Ann Haga

RANK: Capt

BRANCH OF SERVICE: U.S. Air Force

YEAR: 1989

NUMBER OF PAGES: 60

DEGREE: Master of Science in Medical Sciences

INSTITUTION: Department of Pathology and Laboratory Medicine, College of Medicine, University of Florida

THE BIOCHEMICAL CHARACTERIZATION OF PLASMA CLASS I HUMAN LEUKOCYTE ANTIGENS (HLA)

BY

JO ANN HAGA

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

1989

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to the members of my committee, Drs. Kuo-Jang Kao, William J. Riley and Ben M. Dunn, for their encouragement and guidance. I especially thank my mentor and committee chair, Dr. Kao, for his enthusiasm and concern for my educational and career goals as well as his commitment to this research.

I also thank Ms. Becky Spillar for her assistance in obtaining samples, Ms. Sheryl Gresham for HLA phenotyping, and Dr. Nancy Denslow for her advice and expertise in preparation of samples for amino acid sequencing. Special thanks go to Ms. Claudia McQueen for technical assistance, for the training she gave me, and for her friendship.

I would also like to express my appreciation to Dr. Paul Klein and Ms. Linda Green for their training and guidance in the development of monoclonal antibodies and Dr. Claude McGowan for guidance and amino acid analysis of CPY digests.

This project could not have been completed without the generous donation of blood samples by individuals in the department as well as individuals involved in the diabetes studies. My utmost gratitude goes to those individuals and to the families that participated in the pedigree analyses.

I also thank my fellow graduate students for their camaraderie. I especially thank my husband, Kelvin A. Haga, and daughter, Lindsey M. Haga, for their love, understanding and encouragement.

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of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

THE BIOCHEMICAL CHARACTERIZATION OF PLASMA
CLASS I HUMAN LEUKOCYTE ANTIGENS (HLA)

By

JO ANN HAGA

December 1989

Chairman: Kuo Jang Kao
Major Department: Pathology and Laboratory Medicine

Biochemical complexity of plasma HLA antigens was investigated to identify and characterize the different molecular weight (M.W.) forms of HLA in plasma and to derive possible mechanisms for generation of plasma HLA. Additionally, plasma HLA was studied in a representative HLA-phenotyped population (n=44, 51% female) to determine plasma HLA patterns and identify factors which influence the expression of different M.W. forms of plasma HLA.

Gel filtration chromatography of plasma revealed two distinct pools containing HLA. Pool I had apparent molecular weight (M.W.) of 200,000 D and contained only intact 44 kD HLA that was extractable by Triton X-114 detergent. Pool II had M.W. equivalent to 50,000 D and contained 39 and 36 kD HLA which were not extracted by Triton X-114. NH_2-terminal sequence analysis of purified 44 and 39 kD plasma HLA demonstrated identical sequences homologous to the NH_2-terminal consensus sequence of cellular HLA. The NH_2-terminal sequence for the

36 kD HLA was different from the 44 and 39 kD HLA and also unique, as a search against known protein sequences in the database of Genebank revealed no matches. The 36 kD plasma HLA may be a novel Class I-like HLA molecule (non-A, -B, or -C).

These results suggest that 1) only the 44 kD plasma HLA possesses the transmembrane domain and 2) the molecular weight of the 39 kD plasma HLA, lacking the hydrophobic transmembrane domain, precludes that it is generated from cleavage of the cytoplasmic tail and transmembrane domain at the carboxyl terminal. Thus, the 39 kD plasma HLA may represent a secretable form of Class I HLA with deletion of the transmembrane domain through alternative splicing.

Further analysis of plasma HLA of 44 HLA-phenotyped individuals demonstrated a 34 kD plasma HLA in addition to the 44, 39 and 36 kD HLA. Plasmas from all individuals contained 44 and 36 kD HLA but varied as to presence of 39 and 34 kD HLA. Thus, four different plasma HLA patterns were observed in our population. No association between HLA phenotype and absence of 34 kD plasma HLA was seen; however, 7 of 8 individuals without the 39 kD HLA are HLA-B7 positive and female. The exception was an HLA-A1 and B8 homozygous male. Pedigree analysis of families of HLA-B7 positive and 39 kD negative probands further supports that HLA-B7 allele and female gender are necessary but insufficient for the absence of 39 kD HLA in plasma. The results suggest that additional genetic factors may be required to inhibit generation of 39 kD plasma HLA through alternative splicing.

INTRODUCTION

Class I Human Leukocyte Antigens (HLA) are encoded by a highly polymorphic gene complex, the Major Histocompatibility Complex (MHC), located on the short arm of chromosome 6 (1,2). There are three Class I loci, HLA-A, -B and -C (1). Numerous alleles have been identified both serologically and by alloreactive or antigen-restricted cytotoxic T lymphocyte responses for each of the Class I loci (1); currently, 23 HLA-A, 47 HLA-B and 8 HLA-C alleles have been described (2). Class I HLA gene structure consists of 8 exons (3), each generally encoding a structural domain of the protein. Exon 1 encodes a leader peptide; exons 2, 3 and 4, the extracellular globular domains; exon 5, the transmembrane domain; and exons 6, 7 and 8, the cytoplasmic tail (3,4). Class I HLA are codominantly expressed, therefore the phenotype of an individual reflects both parental haplotypes (2).

Functionally, Class I HLA molecules are expressed on the cell surface and are responsible for presenting processed antigen to cytotoxic T lymphocytes (CTL), thereby restricting CTL response to cells identified as self (1,2,5). Additionally, Class I HLA elicit an alloantigenic response resulting in rapid rejection of allografts (1,2). As such, the Class I HLA antigens are referred to as major transplantation or histocompatibility antigens (1,2,5).

Biochemically, Class I molecules are integral membrane glycoproteins (1,5). They are heterodimers, consisting of a 44 kD

glycosylated heavy chain that is noncovalently associated with an invariant 12 kD polypeptide light chain, β-2-microglobulin (β_2m) (1,5,6). The gene encoding β_2m is not located within the MHC on chromosome 6. Instead, it has been mapped to chromosome 15 (7).

Structurally, the heavy chain includes three extracellular domains, a hydrophobic transmembrane domain which anchors the molecule in the cell membrane, and a hydrophilic cytoplasmic tail (1,5). The three extracellular domains are each approximately 90 amino acids in length and are designated as alpha-1 (α-1), alpha-2 (α-2) and alpha-3 (α-3) (1,5,8). The amino-terminal α-1 domain contains an N-linked glycosylation site at asparagine in position 86 (9-11). The carbohydrate moiety, with MW of approximately 3 kD, has no effect on the antigenicity of the HLA molecule (10, 12). The heavy chain contains two linearly arranged intrachain disulfide bonds (13, 14), one in the α-2 domain between cystine residues at positions 101 and 164 and the second in the α-3 domain between cystine residues at positions 203 and 259 (11, 13, 14). While α-1 and α-2 share significant internal structural homology, α-3 shares sequence and structural homology with β_2m and immunoglobulin constant regions (8, 11, 13-15). The transmembrane domain encoded by exon 5 is approximately 40 amino acid residues in length and contains a stretch of 29 nonpolar amino acids which make up the transmembrane region spanning the cell membrane (3). The cytoplasmic tail is hydrophilic in nature and contains several basic residues adjacent to the hydrophobic membrane-binding segment (1, 3, 16, 17). Recent study of X-ray crystallography of HLA-A2 demonstrated

that all three of the extracellular heavy chain domains interact with β_2m and the α-1 and α-2 domains form a groove that binds peptide antigen (18).

In 1970, the presence of Class I HLA in serum was demonstrated by its ability to specifically inhibit anti-HLA antiserum in the lymphocyte microcytotoxicity test (19, 20). In 1973, Aster et al. detected HLA antigen activity in the high density lipoprotein (HDL) fraction of normal plasma prepared by density gradient ultracentrifugal floatation and the alpha-lipoprotein fraction isolated by hydroxylapatite chromatography, as well as, in the plasma and serum of normal subjects (21). Additionally, it was determined that plasma contained significant HLA activity (14%) when compared with the activity of platelets (73%), lymphocytes (6%) and granulocytes (7%) (21). This group also demonstrated elution or shedding of HL-A2 antigen from platelets incubated in HL-A2-negative plasma and suggested that soluble HLA antigens in plasma result from shedding of antigen-lipoprotein from cell membranes (21). Their conclusion that plasma HLA is not present as membrane fragments is supported by the finding that HLA activity in plasma is not affected by centrifugation at 115,000 g for two hours, conditions which would sediment membrane fragments (21).

In 1975, Billing and McMillan confirmed the presence of HLA activity in the HDL fraction of serum prepared by polyanion precipitation of HDL using dextran sulfate and manganese chloride followed by ultracentrifugal floatation (22). Subsequently, Allison et al. (23) isolated intact HLA antigen consisting of a 45 kD heavy chain associated with β2m in HDL fractions of serum prepared by

ultracentrifugal floatation and polyanion precipitation using sodium phosphotungstate and magnesium chloride (23). The MW of the HDL-associated HLA determined by column chromatography was 190 kD (23). These results suggested that the HLA antigens in serum are most likely shed from cell membranes and consist of the HLA heavy chain, β_2m, and lipid noncovalently bound to the transmembrane domain (23).

More recently, Krangel et al. (24) identified an alternatively spliced, secretable form of Class I HLA, with absence of the transmembrane domain, in a mutagenized B lymphoblastoid cell line. Subsequent analysis of cDNA, mRNA, and genomic DNA from the B cell mutant and parent cell line substantiated that alternative splicing, resulting in deletion of exon 5 encoding the transmembrane domain in mRNA transcripts, occurred to produce the secreted form of the molecule in the mutant cell line while normally spliced transcripts produced a cell-associated, intact molecule in both cell lines (25).

Secretable HLA antigens of both HLA-A and -B specificity were subsequently demonstrated in T leukemic cell lines (26). Conversely, allele-specific secretion of HLA-A9 and HLA-A24 (a serological split of HLA-A9) was noted in a hepatoma cell line and a B lymphoblastoid cell line, respectively (26). HLA-A9 and HLA-A24 were also associated with increased levels of water-soluble antigen in other cell lines (26). Thus, secretable HLA antigens in various cell lines may consist of products of both HLA-A and -B loci or they may consist of the product of a single allele, in this case, HLA-A9 or -A24. Additionally, relative quantity of secretable HLA antigens may be associated with a particular allele.

Subsequent in vitro evaluation of mitogen-activated peripheral blood lymphocytes from three donors also demonstrated secretable HLA-A and -B antigens (26). These results suggested that secretion of HLA-A and HLA-B antigens occurs in tumor cells and normal cells in vitro and that alternative splicing may be the mechanism responsible for generation of soluble antigens in normal cells as well as in the mutant B lymphoblastoid cell line described (26).

Further studies conducted in 1987 demonstrated the presence of 43 kD and 39 kD HLA in plasma. The 39 kD plasma HLA had immunochemical properties identical to the alternatively spliced 39 kD molecule detected in vitro (27). Among individuals tested, those with HLA-A24 phenotype consistently demonstrated increased levels of 43 and 39 kD plasma HLA (27).

Recently, Dobbe et al. reported more complex patterns of Class I HLA in serum (28). Three to seven different forms of HLA, ranging in molecular weight from 44 to 35 kD, were observed, with 44, 40, 37 and 35 kD HLA predominating (28). Since the 40 kD HLA in most of the sera tested did not react with an antiserum directed against the cytoplasmic tail of Class I HLA, they concluded that alternative splicing is not the primary mechanism responsible for generation of the 40 kD plasma HLA (28). Although carboxyl terminal antisera would be expected to react with alternatively spliced HLA molecules, it was noted that alternative splicing could result in a shift in the DNA reading frame, thereby producing an alternatively spliced HLA with a cytoplasmic tail different from that of the intact molecule (28). The cytoplasmic tail of molecules spliced in this manner would probably not be recognized by the

antiserum. Additionally, as the 35 and 37 kD HLA did not contain the cytoplasmic tail or transmembrane domain, it was suggested that they were proteolytic products of intact 44 kD HLA (28). An association between HLA-A24 and increased levels of 44 and 40 kD Class I HLA in serum was also noted in their study (28). Furthermore, the presence of both 37 and 35 kD HLA in the plasma of one family was found to be associated with HLA-A28 (28).

Evaluation of plasma HLA concentration in 215 individuals by Kao et al., using an enzyme-linked immunoassay (EIA), also revealed significantly increased levels of total plasma HLA in individuals with HLA-A23 and HLA-A24 (both serological splits of HLA-A9), while HLA-A26 was associated with decreased levels of plasma HIA (29). In contrast, HLA-A23 and HLA-A24 were not associated with increased platelet HLA (29). These results suggested that expression of platelet and plasma HLA may be regulated differently (29).

It is apparent that several molecular weight forms of Class I HLA are present in serum and plasma and that both quantitative and qualitative expression of plasma HLA varies between individuals and may depend, to some extent, on HLA phenotype. Several groups, in particular, have noted the association between HLA-A9 (HLA-A23 and HLA-A24) and increased levels of plasma HLA (22, 23, 27-29).

In addition to the probable associations between HLA phenotype and expression of plasma HLA, a consensus regarding the number of different molecular weight forms of Class I HLA in normal plasma has not been reached. The earliest report (23) indicated there was only one form of HLA in the HDL fraction of serum with molecular weight of 44-45 kD, the

same as intact cellular antigen. Subsequently, a water-soluble, 39 kD HLA was detected in plasma in addition to the intact, lipid-associated 44 kD HLA (27). This was followed by the identification of additional water-soluble 37 and 35 kD HLA in plasma (28). Formal studies to determine the number and distribution of different molecular weight forms of plasma HLA in a large number of individuals have not been completed.

Regarding the mechanisms by which plasma HLA is generated, it has been suggested that membrane shedding (21, 23) is responsible for the appearance of the intact 43-44 kD HLA in plasma and that degradation of the intact 44 kD molecule is responsible for the generation of the lower molecular weight forms (35-37 kD) of plasma HLA (28). Alternative splicing during mRNA transcription and processing resulting in transmembrane deletion and generation of a secreted form of HLA may be the mechanism responsible for the 39-40 kD plasma HLA (26, 27). Although evidence provided by Dobbe et al. (28) does not support alternative splicing as the mechanism primarily responsible for generation of the 39-40 kD plasma HLA, it does not disprove it. Further direct biochemical evidence is necessary to demonstrate that 39-40 kD plasma HLA is generated from alternative splicing <u>in vivo</u>.

In this study, we identified and characterized the different molecular weight forms of Class I HLA present in plasma to further elucidate the mechanisms by which plasma HLA is generated. Additionally, plasma HLA patterns in an HLA-phenotyped population were evaluated and the possible correlation between plasma HLA patterns and HLA phenotype was investigated.

MATERIALS AND METHODS

Subjects Included in the Study

A total of forty-four HLA-phenotyped individuals, 31 healthy volunteers and 13 individuals with type I diabetes followed at the Clinical Research Center at the University of Florida, were available for the study to determine the presence of different molecular weight forms of HLA in plasma. Twenty-one were male and twenty three were female. The age of individuals included in the study ranged from 6 to 53 years. The median age was 23.5 years and the mean age was 23.4 years. As reported previously, there is no significant difference in plasma HLA concentration in type I diabetics compared with healthy volunteers (29).

Preparation of Plasma Samples

Venous blood was drawn into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (Becton-Dickinson, Rutherford, NJ). The top two-thirds of plasma was harvested after centrifugation of venous blood at 2,000 g for 10 min at room temperature. Protease inhibitors, phenylmethylsufonyl fluoride (PMSF) (Sigma Co., St. Louis, MO) and diisopropylphosphofluoridate (DFP) (Sigma) were added to plasma at a final concentration of 1 mM. Stock PMSF was prepared in 100% ethanol at a concentration of 100 mM. After

addition of protease inhibitors, plasma samples were stored at -70°C until analysis.

Monoclonal Antibodies (MoAb)

W6/32 Monoclonal Antibody

W6/32 anti-HLA monoclonal antibody (MoAb) is an IgG_2a mouse-anti-human MoAb which is reactive against a monomorphic epitope present on Class I HLA antigens (30-32). Association of β_2m with HLA heavy chain is essential for the expression of this epitope (32). W6/32 hybridoma cells were cultured in RPMI-1640 media (GIBCO Lab, Grand Island, NY) containing 10% newborn calf serum (GIBCO), 1% antibiotic-antimycotic solution (Sigma) and 0.125% gentamicin (Sigma). To obtain W6/32 MoAb in quantities of milligrams, each female Balb/c mouse was primed with 2,6,10,14-tetramethylpentadecane and injected with 5 X 10^6 cells suspended in 0.5 ml of sterile Hemagglutination Buffer, pH 7.3 (HAB) (Difco) as described (33). Ascites fluid was collected and centrifuged at 2,000 g for 10 min to remove contaminating cellular elements and the fibrin clot. The antibody was subsequently purified by using protein A affinity column chromatography (33). The isolated W6/32 MoAb appeared to be homogeneous as judged by SDS-PAGE and was used for immunodetection of HLA, immunoprecipitation of HLA and affinity purification of HLA. These procedures will be described individually.

HC-10 Monoclonal Antibody

HC-10 anti-HLA heavy chain (HC) MoAb is an IgG_2a mouse anti-human antibody which is reactive against HLA HC with marked preference for

HLA-B locus products (34). HC-10 also reacts with HLA-A3, A33 and A29. It reacts less well with HLA-A1, A2, A11 and A24 and recognizes additional Class I polypeptides that correlate with HLA-C locus products (34). This antibody was a generous gift from Dr. HL Ploegh and was used for immunodetection of HLA HC on Western Blot.

Rabbit Anti-HLA Heavy Chain Antibody (anti-HLA HC)

The polyclonal rabbit anti-HLA HC antisera was a gift from Dr. HL Ploegh. It was used for detection of HLA antigens in plasma fractions.

21.1 Anti-thrombospondin Monoclonal Antibody

21.1 anti-thrombospondin MoAb was obtained from Dr. KJ Kao and was used as a negative control for immunodetection of HLA on Western Blot.

Gel Filtration Column Chromatography of Plasma HLA

Five milliliters of plasma harvested from venous blood anticoagulated with EDTA was fractionated with a 480 ml Sephacryl S-300 (Sigma) column (2.6 cm X 90 cm) at a flow rate of 25 ml per hour. The column was equilibrated with PBS-azide. Fractions of approximately 3 ml were collected using an LKB 2112 Redirac Fraction Collector (LKB Instruments, Gaithersburg, MD). The absorbance of each fraction at 280 nm was measured with an LKB Ultrospec 4050 spectrophotometer. Presence of HLA antigens in each fraction was then measured by an enzyme-linked immunoassay (EIA). For estimation of molecular weight, the column was calibrated with the following protein markers, each at an approximate concentration of 2 mg/ml in PBS-azide: Blue Dextran (2,000 kD), β-

amylase (200 kD), alcohol dehydrogenase (150 kD), carbonic anhydrase (29 kD), and cytochrome C (12.4 kD) (Sigma).

Enzyme-Linked Immunoassay (EIA) for HLA

To detect HLA antigens in plasma fractions, a polystyrene immunoassay plate (NUNC Inter-Med, Kamstrup, Denmark) was coated overnight at room temperature with 100 ul of W6/32 anti-HLA MoAb (5 ug/ml) in PBS-azide. The plate was washed three times with plate washing buffer (PBS-azide containing 0.5% Tween-20) and blocked with 200 ul of PBS-azide with 1% bovine serum albumin (BSA) and 0.5% Tween-20 for 30 min at room temperature. After three washes, 100 ul of plasma fractions obtained from gel filtration chromatography was added to each well and incubated for 90 min at room temperature. After washing three times, 100 ul of rabbit anti-HLA HC diluted 1000X in PBS-azide containing 1% BSA and 0.5% Tween-20 was added and incubated at room temperature for 60 min. Subsequently, 100 ul of goat anti-rabbit IgG whole molecule alkaline phosphatase conjugate (1:1000 in PBS-azide containing 1% BSA and 0.5% Tween-20) was added and incubated for 30 min at room temperature. The plate was again washed three times and 200 ul of alkaline phosphatase substrate (1 mg/ml p-nitrophenylphospate disodium) was added and allowed to incubate 30-60 min at room temperature. Absorbance was measured at 405 nm in a Molecular Devices Vmax microplate reader (Molecular Devices, Palo Alto, CA).

Immunoprecipitation of Plasma HLA

Routinely, plasma HLA was immunoprecipitated with W6/32 MoAb coupled to cyanogen bromide-activated Sepharose 4B gel (Sigma). The Sepharose 4B bound W6/32 was prepared as described (35). Normally, every 3 mg W6/32 MoAb was coupled to 1 ml of CNBr-activated gel with >97% coupling efficiency.

Immunoprecipitation of HLA was accomplished by incubation of sample with appropriate quantities of antibody coupled to sepharose beads overnight at 4°C. For plasma samples, each milliliter of plasma was incubated with solid phase W6/32 containing approximately 12 ug of antibody. Thereafter, the gel was washed 4 times with PBS-azide and eluted with Laemmli sample buffer for SDS-PAGE.

HLA for NH_2-terminal sequencing was purified from 50 ml of plasma by immunoprecipitation as described, however an additional wash with 0.01 M phosphate buffer containing 0.5 M NaCl was added. HLA was then eluted from the gel with 0.05 M diethylamine, pH 11.5 containing 0.1% NP-40, and dialyzed against PBS-azide with 0.05% SDS overnight at 4°C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The procedure of SDS-PAGE was essentially the same as reported by Laemmli (36). Molecular weight markers used included myosin (200 kD), phosphorylase b (97.4 kD), BSA (66 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD), β-lactoglobulin (17.85 kD), and lysozyme (15.1 kD).

Immunoblot

After SDS-PAGE, gels were electrophoretically transferred to PVDF membranes (37) prepared by wetting in 100% methanol and incubation in blot transfer buffer (0.025 M Tris-base, 0.192 M glycine, 20% methanol, 0.1% SDS, pH 8.3) for at least 30 min at room temperature. Transfer was performed at 50 V constant voltage for 3 hours. The membrane was then blocked in 5% nonfat milk in PBS-azide for 2 hours at room temperature, then washed three times with PBS-azide, 0.05% Tween-20 for 10 minutes each. After washing, the membrane was incubated with HC-10 anti-HLA-HC MoAb (1:1000 in PBS-azide, 1% bSA, 0.05% Tween-20) overnight at room temperature. The membrane was washed as described above and incubated with rabbit anti-mouse IgG-alkaline phosphatase conjugate (1:1000 dilution) for 2 hours at room temperature. The membrane was washed three times again, rinsed for 5 min in substrate buffer (0.1 M Tris-HCl, 1 mM $MgCl_2$, pH 8.8), and developed with alkaline phosphatase substrate [0.1 mg/ml nitro-blue tetrazolium (Sigma) and 0.05 mg/ml 5-bromo-4 chloro-3-indolyl phosphate (Sigma) in substrate buffer]. The developed membrane was rinsed in water prior to air drying. Alternately, membranes were rinsed in distilled water for 5 min at room temperature immediately after transfer and stained with 0.1% Coomassie Blue R-250 (Serva) in 50% methanol for 5 min. Then the membranes were destained in two changes of 50% methanol containing 10% acetic acid for 5 min each, and rinsed in distilled water prior to air drying (38).

In vitro Degradation Studies

In vitro degradation of plasma HLA was investigated in whole blood collected with vacutainers containing EDTA or acid citrate dextrose (ACD) anticoagulant. Venous blood was aseptically divided into five 3 ml aliquots, and incubated at room temperature. At 0, 6, 12, 24 and 48 hours, whole blood aliquots were centrifuged at 2,000 g for 10 min. PMSF was added to the plasma sample to a final concentration of 1 mM. The plasma samples were immunoprecipitated with W6/32 anti-HLA MoAb as described. The eluates were stored frozen at -20°C until analysis by SDS-PAGE and immunoblot.

In vitro degradation of plasma HLA was also evaluated in plasma samples aliquoted and incubated at 37°C. Plasma samples were analyzed as described for whole blood at 0, 24, 48 and 72 hours.

Affinity Column Purification of Plasma HLA

Plasma HLA for NH_2-terminal sequencing was purified using a column prepared with W6/32 anti-HLA MoAb coupled to CNBr-activated Sepharose 4B as described by Parham (39). A normal mouse IgG column was used to pre-clear the plasma prior to application to the W6/32 column. Both columns were pre-eluted with 0.05 M DEA, pH 11.5, followed by washing with PBS-azide containing 0.5 M NaCl and finally with PBS-azide. A unit of HLA-phenotyped fresh frozen plasma, previously stored at -70°C, was thawed in a 37°C water ba and centrifuged at 10,000 g for 30 min. HLA in 365 ml of plasma was isolated by column chromatography at 4°C on the sequentially-linked normal mouse IgG and W6/32 columns. The W6/32 column was washed at 4°C overnight with PBS-azide, followed by washing

with 100 ml of PBS-azide containing 0.5 M NaCl and with 50 ml of PBS-azide containing 0.15 M NaCl. The column was eluted with 0.05 M diethylamine (DEA), pH 11.5. The eluted protein peak containing HLA was identified by assaying 20 ul of each fraction with a coomassie dye-binding method (Biorad Micro Protein Assay). The protein peak was pooled and dialyzed into PBS-azide overnight at 4°C. Following dialysis, the HLA was concentrated times two using an Amicon Centriprep Concentrator. The protein concentration was subsequently measured using the micro method of Lowry et al. (40).

NH_2-terminal Sequence Analysis

Plasma HLA purified by affinity column chromatography or immunoprecipitation from two HLA-phenotyped individuals was separated by SDS-PAGE and electrophoretically transferred to Immobilon as described. However, a blot transfer buffer that did not contain glycine was used in order to eliminate background interference of glycine during amino acid sequencing. The blot transfer buffer used was 10 mM MES (2[N-morpholino]ethanesulfonic acid), pH 6.0 containing 20% methanol and 0.01% SDS. All reagents used for SDS-PAGE, blotting and staining of the membrane were prepared in acid-washed glassware. Gloves were worn throughout these procedures to avoid contamination of the membrane-bound proteins.

After transfer, the membrane was washed, stained with 0.1% Coomassie Blue R-250 (Serva) in 50% methanol and destained with a solution of 50% methanol and 10% acetic acid as described previously. This was followed by 5 rinses in distilled water at room temperature for

5 min each. The membrane was air dried on clean plastic wrap with a loose foil cover. After drying the membrane was wrapped in clean plastic wrap, sealed in foil, and submitted to the Protein Core Laboratory at the University of Florida for NH_2-terminal sequencing. The membrane was stored at -20°C until sequencing analysis.

For NH_2-terminal sequencing, stained bands were excised from the membrane with a clean scalpel. The membrane was placed in the sample cartridge block of a model 470A Sequenator (Applied Biosystems). Phenylthiohydantoin (PTH) derivatives of amino acids were identified with an on-line 120A PTH Analyzer (Applied Biosystems), using reverse phase HPLC.

Triton X-114 (TX-114) Extraction of Plasma HLA

Plasma was extracted with TX-114 essentially as described by Bordier (41) and by Dobbe et al. (28). EDTA plasma (3.0 ml) was first diluted 20-fold in ice-cold PBS-azide and divided into 2 aliquots of equal volume. One aliquot was reserved for analysis of plasma HLA prior to extraction. The other aliquot was made 1% with precondensed TX-114, incubated for 2 hours at 4°C, and subjected to phase separation by incubation at 37°C for 5 min. TX-114 was pelleted by centrifugation at 2,000 X g for 10 min at room temperature. Each plasma sample was extracted with TX-114 three times. Detergent phases were pooled, diluted to the initial aliquot volume in PBS-azide, and maintained in solution at 4°C. The aqueous supernatants and the TX-114 detergent phases were immunoprecipitated with W6/32 and analyzed by SDS-PAGE and immunoblot as described.

Papain Digestion of Purified HLA

HLA purified from platelets was digested with solid-phase papain (Sigma) for comparison of the papain-digested product to plasma HLA. Briefly, 250 ug of purified HLA was dialyzed against 400 ml of 0.01 M Tris-HCl, pH 8.0, containing 2 mM EDTA. Solid-phase papain (1 mg) was incubated in 1.5 ml distilled water for 2 hours on ice, then washed three times with 1.5 ml 0.01 M Tris-HCl, pH 8.0, containing 2 mM EDTA, and resuspended in 100 ul of the same buffer. Twenty microliters of the solid-phase papain (14 ug) was added to 250 ug of purified HLA, vortexed gently every few minutes at room temperature. Thereafter, aliquots containing 50 ug of HLA (65 ul) were removed at 2 min, 5 min, 10 min, 20 min, and 60 min, and immediately microfuged. The supernatant was transferred to clean microtubes. An equal volume of Laemmli sample buffer was added and the samples were heated in a boiling water bath for 2 min. Samples were frozen at -20°C until analyzed by SDS-PAGE.

Lymphocyte Microcytotoxicity Assay

HLA-phenotyping of individuals included in the study was performed by the HLA Typing Laboratory in the Department of Pathology and Laboratory Medicine at the J. Hillis Miller Health Science Center at the University of Florida. HLA phenotyping was accomplished using the standard lymphocyte microcytotoxicity assay (42).

Pedigree Analysis

Plasma samples from members of 4 families were obtained. Distribution of different molecular weight forms of plasma HLA was studied by using immunoprecipitation, SDS-PAGE and immunoblot as described.

RESULTS

Gel Filtration Chromatography of Plasma HLA

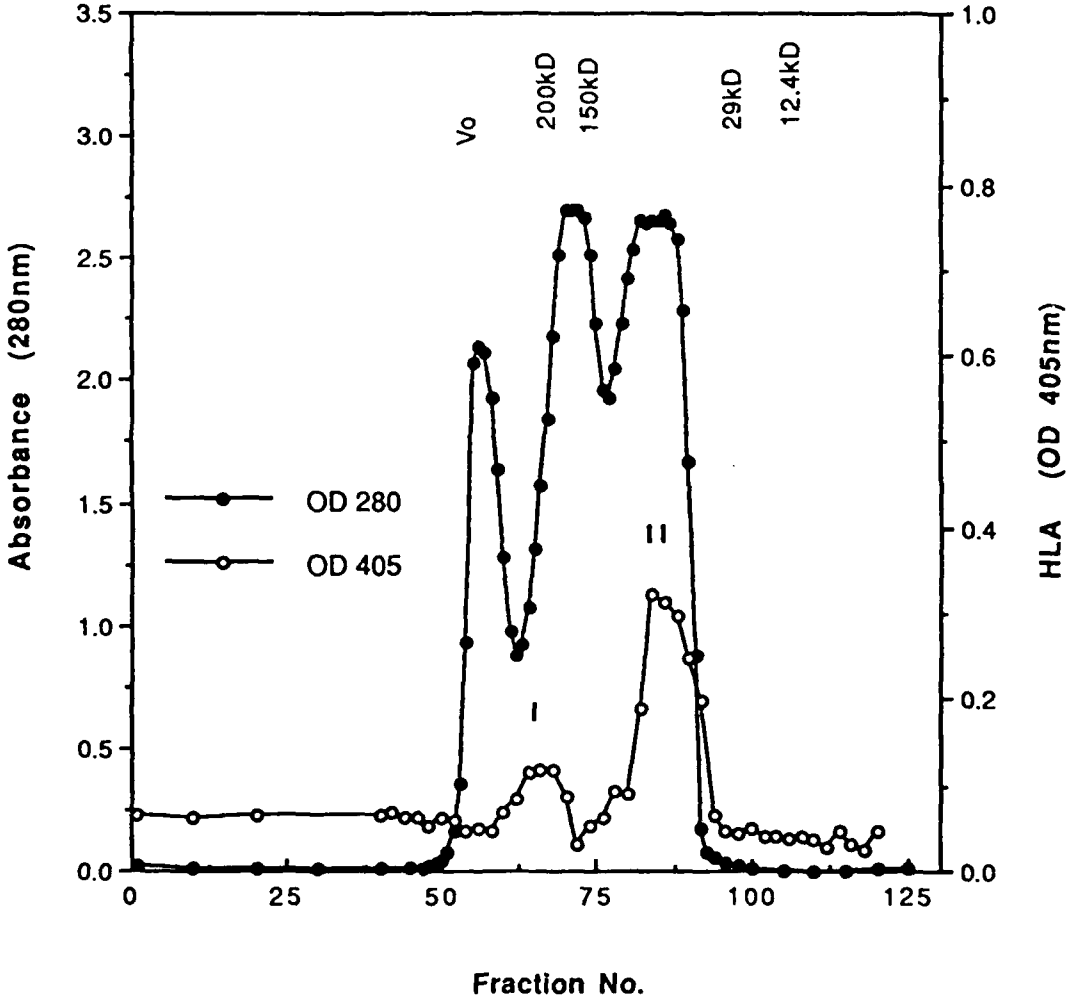
Previously, Allison et al. (23) detected a single HLA peak with a molecular weight of approximately 190 kD in the HDL fraction of serum by gel filtration chromatography. Papain digestion of HDL-associated HLA (which results in removal of the hydrophobic, lipid-binding transmembrane domain), followed by column chromatography resulted in separation of the delipidated HLA from HDL at an approximate molecular weight of 46 kD (23).

In order to determine the apparent molecular weight (M.W.) of HLA in plasma, plasma samples were fractionated by gel filtration chromatography on a calibrated Sephacryl S-300 column. HLA antigens in each fraction were then detected by an enzyme-linked immunoassay (EIA).

As shown in Figure 1, fractionation of plasma HLA results in the separation of two different molecular weight pools, designated as I and II. The same finding was observed in plasma samples from three different individuals. However, the relative quantities of HLA in pool I and II varied among individuals.

Pool I HLA has an apparent M.W. of 200 kD and therefore, most likely corresponds to the lipid-associated HLA detected by Allison (23). Pool II HLA has an approximate M.W. of 50 kD, similar to that of the 46

Figure 1. Fractionation of Plasma HLA by Sephacryl S-300 Column Chromatography. EDTA plasma samples were fractionated by gel filtration chromatography on a calibrated Sephacryl S-300 column. The absorbance of each fraction at 280 nm and the OD at 405 nm of HLA measured by EIA are plotted on the vertical axis against the fraction number on the horizontal axis. Two separate pools of plasma HLA are identified: pool I at M.W. 200,000 D and pool II at M.W. 50,000 D. M.W. markers: Blue Dextran (V_o), β-amylase (200 kD), alcohol dehydrogenase (150 kD), carbonic anhydrase (29 kD), and cytochrome C (12.4 kD).


Absorbance (280nm)
HLA (OD 405nm)
Fraction No.
OD 280
OD 405
Vo
200kD
150kD
29kD
12.4kD
I
II
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.0
0.8
0.6
0.4
0.2
0.0
0
25
50
75
100
125

kD proteolytic fragment of HLA separated by column chromatography from HDL (23).

SDS-PAGE of Pool I and Pool II Plasma HLA

Plasma HLA in pool I and pool II was then immunoprecipitated with W6-32 anti-HLA MoAb coupled to sepharose beads. Immunoprecipitated HLA was analyzed by SDS-PAGE. The molecular weight of immunoprecipitated HLA polypeptides on SDS-PAGE was then determined by using immunoblot with HC-10 anti-HLA HC MoAb. As shown in Figure 2, pool I consisted entirely of intact 44 kD HLA while pool II contained two different molecular weight forms of HLA, 39 kD and 36 kD.

The molecular weight of HLA in pool I coincides with that of HDL-associated HLA detected by Allison et al. (23), which consisted of a 45 kD heavy chain and β2m. As suggested by both Aster et al. (21) and Allison et al. (23), this form of plasma HLA is probably lipid-associated and may be shed from cell membranes.

In vitro Degradation of HLA in Whole Blood and Plasma

In order to identify whether low M.W. forms of plasma HLA are derived from proteolytic degradation of intact HLA, quantitative distribution of different M.W. forms of plasma HLA was studied in plasma and whole blood stored in vitro for various durations. As shown in Figure 3, the proportional amount of different M.W. forms remained unchanged in plasma even after 72 hours incubation at 37°C. Moreover, the plasma of individual B (Figure 3) did not contain 39 kD HLA and no detectable amount of 39 kD HLA was generated. Similar findings were

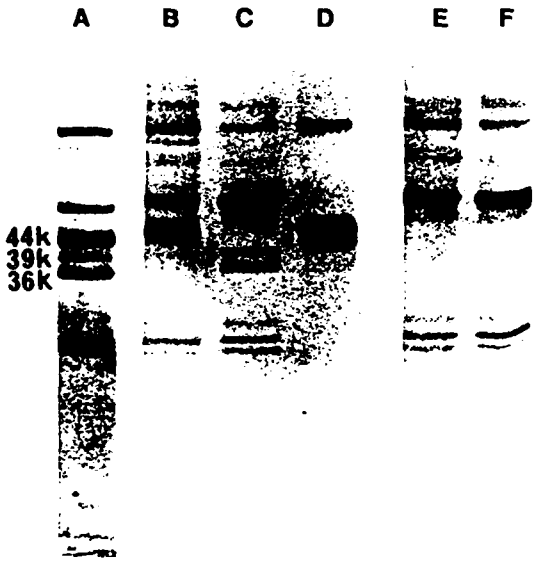


Figure 2. SDS-PAGE of Pool I and Pool II Plasma HLA. HLA in pool I and pool II was immunoprecipitated with W6/32 anti-HLA MoAb and analyzed by SDS-PAGE and immunoblot with HC-10 anti-HLA Heavy Chain MoAb. Pool I consists of a 44 kD HLA heavy chain and pool II consists of 39 and 36 kD HLA heavy chains. Lightly staining bands at approximately 90 kD, 50 kD, and 25 kD are immunoglobulin heavy and light chain dimers, immunoglobulin heavy chains and immunoglobulin light chains, respectively. The immunoglobulin chains are contaminants from the mouse MoAb, W6/32, which is detected by the second antibody used in immunoblotting, rabbit anti-mouse IgG whole molecule-alkaline phosphatase conjugate.

Lane A: Plasma HLA before fractionation
Lane B: Pool I HLA
Lane C: Pool II HLA
Lane D: Purified HLA antigens
Lanes E and F: Pool I and pool II controls immunoprecipitated with W6/32. Immunoblot was performed using 21.1 anti-thrombospondin MoAb instead of HC-10.

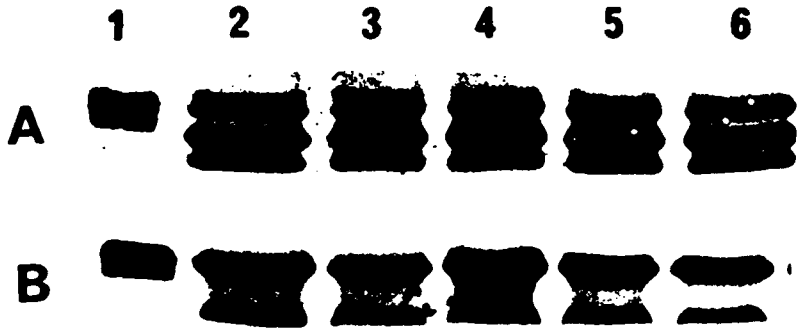


Figure 3. In vitro Degradation of HLA in Whole Blood and Plasma. Venous blood in EDTA was aseptically collected, aliquoted, and stored at room temperature up to 48 hours. Protease inhibitors were not added. At specified times, a sample of plasma was prepared and immunoprecipitated with W6/32 anti-HLA MoAb, followed by SDS-PAGE and immunoblot with HC-10 anti-HLA Heavy Chain MoAb. Degradation of HLA in plasma incubated at room temperature and 37°C was also investigated.

A: Whole Blood

Lane 1: Purified Platelet HLA
Lane 2: 0 hr
Lane 3: 6 hrs
Lane 4: 12 hrs
Lane 5: 24 hrs
Lane 6: 48 hrs

B: Plasma

Lane 1: Purified Platelet HLA
Lane 2: 0 hr
Lane 3: 24 hrs at room temperature
Lane 4: 24 hrs at 37°C
Lane 5: 48 hrs at 37°C
Lane 6: 72 hrs at 37°C

observed when whole blood anticoagulated with ACD was incubated *in vitro* for 2 days (not shown). Thus, it is unlikely that low M.W. forms of HLA are generated through proteolysis of intact HLA in blood.

Patterns of Different M.W. HLA in Plasma of the General Population

Since there are different molecular weight forms of HLA in plasma, we then investigated the possible distributions of various forms of plasma HLA in the general population. Plasma samples from 44 individuals were studied. Four different molecular weight forms of plasma HLA were observed: 44, 39, 36, and 34 kD (Figure 4). The plasma of all individuals studied contained 44 kD and 36 kD HLA but varied as to the presence of 39 kD and 34 kD HLA. Thus, four different patterns of plasma HLA were identified (Figure 4). Furthermore, when the distribucion of different M.W. forms of HLA in plasma of 4 individuals was followed over a year, no change in distribution patterns was observed.

The frequency of each plasma HLA pattern is listed in Table 1. In the population studied, 61% of individuals (n=27) do not have the 34 kD plasma HLA and 18% of individuals (n=8) do not have the 39 kD plasma HLA.

NH_2-terminal Sequence Analysis of Plasma HLA

To further characterize the different M.W. forms of plasma HLA biochemically, the NH_2-terminal amino acid sequence of each molecular weight form was determined. The NH_2-terminal amino acid sequences of plasma HLA were then compared to the NH_2-terminal sequence of cellular

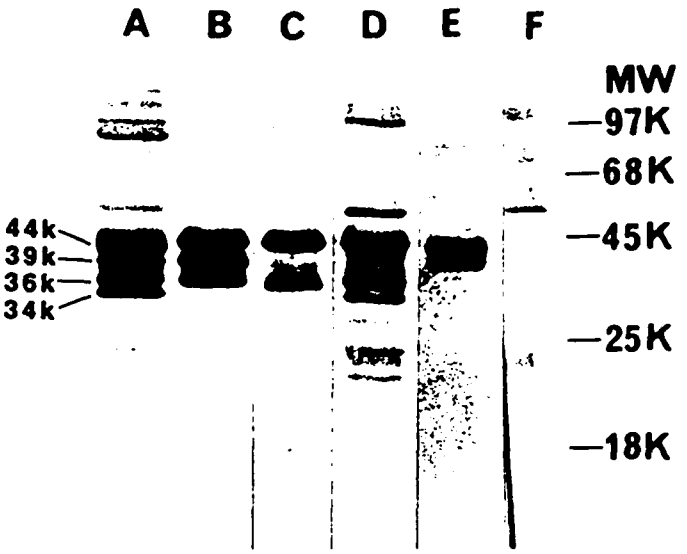


Figure 4. Patterns of Different Molecular Weight Forms of Class I HLA in Plasma. Different forms of plasma HLA were separated by SDS-PAGE followed by immunoblotting with HC-10 anti-HLA Heavy Chain MoAb. M.W. markers: phosphorylase b (97 kD), BSA (68 kD), ovalbumin (45 kD), carbonic anhydrase (25 kD) and β-lactoglobulin (18 kD).

Lane A-D: Plasma HLA
Lane E: Purified HLA antigens
Lane F: Control plasma immunoprecipitated with W6/32 anti-HLA MoAb. Immunoblotting was performed using 21.1 anti-thrombospondin MoAb instead of HC-10 anti-HLA Heavy Chain MoAb.

HLA antigens. As shown in Table 2, only the NH_2-terminal sequences of the 44 kD and 39 kD plasma HLA are identical to that of cellular HLA. The NH_2-terminal amino acid sequence of the 36 kD protein was found to be unique, as the matched sequence could not be found in the database of Genebank. The same NH_2-terminal sequence for the 36 kD HLA was identified when the 36 kD plasma HLA from another unrelated individual was studied. Because the 34 kD HLA was not present in the plasma of either individual studied, the NH_2-terminal sequence for 34 kD HLA was not performed.

Triton X-114 (TX-114) Extraction of Plasma HLA

In order to determine if 39 kD plasma HLA was derived from alternative splicing of messenger RNA with deletion of the transmembrane domain as suggested previously (27), plasma samples were extracted with the nonionic detergent, TX-114. TX-114 has been used previously to separate integral membrane proteins containing a hydrophobic domain from hydrophilic proteins which show little or no hydrophobic interaction with nonionic detergents (41).

HLA was immunoprecipitated from the aqueous and detergent phases with W6/32 anti-HLA MoAb and analyzed using SDS-PAGE and immunoblot. Three individuals' plasmas were evaluated. Only the 44 kD plasma HLA could be extracted into the detergent phase (Figure 5). Both the 39 and 36 kD plasma HLA were excluded from TX-114 and remained in the aqueous phase. The results confirmed the early finding (27) and suggest that only the 44 kD plasma HLA contains the hydrophobic transmembrane domain.

Table 1. Frequency of Different Plasma HLA Patterns Among 44 Individuals.

	Plasma HLA Pattern[a]			
	A	B	C	D
Number	15	21	6	2
%	34.1	47.7	13.6	4.6

[a]See Figure 6 to identify each pattern.

Table 2. NH_2-Terminal Sequences of Plasma HLA

Cellular HLA[a]	Gly-Ser-His-Ser-Met-Arg-Tyr-
44 kD plasma HLA[b]	Gly-Ser-His-Ser-Met-Arg-Tyr-
39 kD plasma HLA[c]	Gly-Ser-His-Ser-Met-Arg-Tyr-
36 kD plasma HLA[d]	Leu-Gln-Ala-Ala-Asp-Thr-

[a]The NH_2-terminal sequence of cellular HLA is highly conserved among alleles of all three of the Class I loci(15).
[b]Approximately 9-10 pmol of protein was present on the PVDF membrane as estimated from sequence results.
[c]approximately 6-7 pmol of protein
[d]approximately 15 pmol of protein

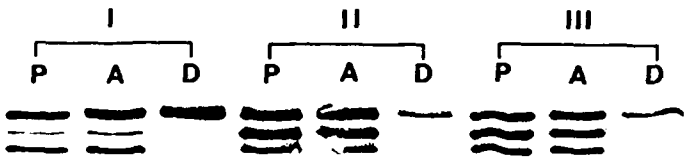


Figure 5. Triton X-114 (TX-114) Extraction of Plasma HLA. Plasma of three individuals was extracted with TX-114. HLA was immunoprecipitated from plasma, aqueous and detergent phases using W6/32 anti-HLA MoAb, followed by SDS-PAGE and immunoblot using HC-10 anti-HLA Heavy Chain MoAb. Only the 44 kD form of plasma HLA binds detergent and is extracted.

P: Plasma HLA before TX-114 extraction
A: HLA remaining in the aqueous phase
D: HLA extracted into the detergent phase

Comparison of 39 kD Plasma HLA With Papain-Digested HLA

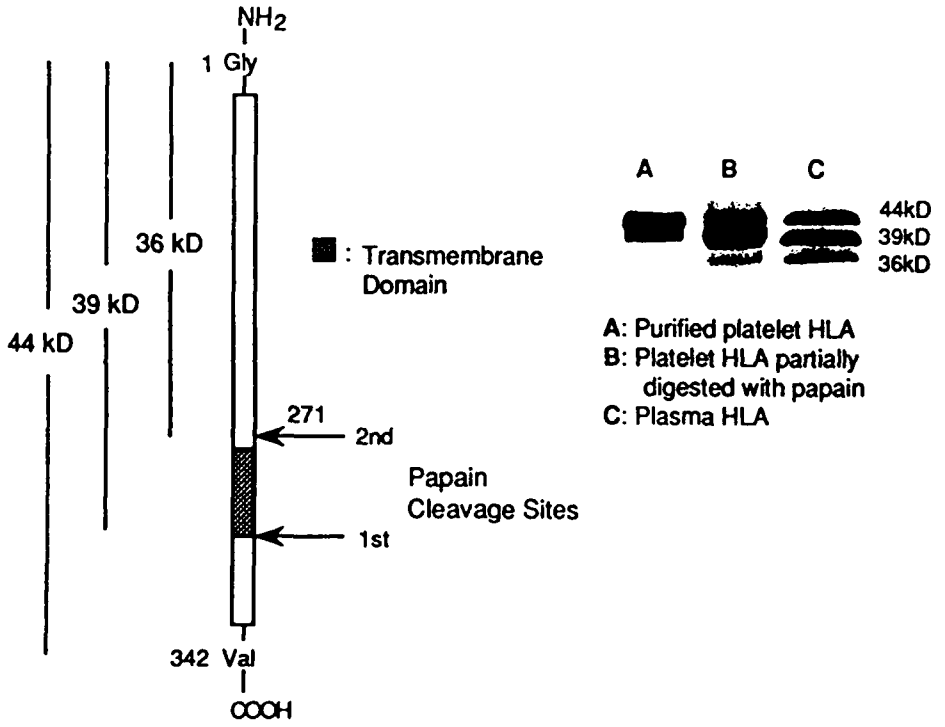
As reported earlier (16, 43), papain digestion of purified cellular HLA first resulted in release of the cytoplasmic tail of HLA antigen with subsequent reduction of the molecular weight of HLA antigen from 44 kD to 39 kD. The 39 kD HLA generated from papain digestion has an intact transmembrane domain and is extractable by TX-114 (26). Further digestion of the 39 kD HLA cleaves the peptide bond at amino acid residue 271 and produces a 36 kD peptide that does not contain the hydrophobic transmembrane domain (43). Comparison of papain-digested platelet HLA with plasma HLA demonstrates the same molecular weight for proteolytic products of intact HLA and the lower molecular weight forms of plasma HLA (Figure 6). Since the 39 kD plasma HLA, unlike papain-digested HLA, could not be extracted by TX-114 (Figure 5), the 39 kD plasma HLA can not be produced through the loss of the cytoplasmic tail of intact HLA antigens. Thus, it is most likely that the 39 kD plasma HLA is produced through alternative splicing with deletion of the transmembrane domain as suggested previously (27).

Evaluation of Plasma HLA in HLA-Phenotyped Individuals

In view of the fact that quantitative expression of plasma HLA was reported to be influenced by HLA phenotype (22, 23, 27-29), we then investigated the possible association of HLA phenotype with various patterns of different M.W. forms of plasma HLA. Forty-four HLA-phenotyped individuals were studied. The antigen frequencies of HLA-A and -B phenotypes among individuals included in our study were similar to the published antigen frequency for American Caucasians, with the

Figure 6. Comparison of Papain-Digested Purified Platelet HLA with Plasma HLA. Purified platelet HLA was partially digested with papain and analyzed with plasma HLA by SDS-PAGE and immunoblot with HC-10 anti HLA Heavy Chain MoAb. The figure on the left illustrates the proteolysed products obtained from papain digestion. Papain initially cleaves the cytoplasmic tail from the intact 44 kD HLA, producing a 39 kD peptide which retains the transmembrane domain. Further digestion releases the transmembrane domain, resulting in the formation of a 36 kD peptide. SDS-PAGE of papain-digested, purified platelet HLA along side plasma HLA demonstrates the similarity in molecular weight of the proteolytic products from papain digestion of platelet HLA and the lower molecular weight forms of plasma HLA.

Lane A: Purified platelet HLA
Lane B: Platelet HLA partially digested with papain
Lane C: Plasma HLA


NH2
1 Gly
36 kD
39 kD
44 kD
: Transmembrane Domain
271
2nd
Papain Cleavage Sites
1st
342 Val
COOH
A
B
C
44kD
39kD
36kD
A: Purified platelet HLA
B: Platelet HLA partially digested with papain
C: Plasma HLA

exception of a few phenotypes (Table 3). A much higher number of HLA-B62 positive individuals in our study is due to the increased occurrence of this antigen in type I diabetics (44, 45, 46).

Correlation of HLA Phenotype and Gender With Plasma HLA Patterns

As described earlier, four different patterns of plasma HLA were identified among individuals included in the study (Figure 4). Correlation of the four different patterns of plasma HLA with HLA phenotypes reveals that only absence of 39 kD plasma HLA is significantly associated with HLA-B7 ($p<0.00015$) (Figure 7). Seven of 8 individuals who did not have the 39 kD plasma HLA were positive for HLA-B7. Due to linkage disequilibrium between HLA-A3 and HLA-B7 (47), the percentage of HLA-A3 positive individuals was also increased among those with absence of 39 kD plasma HLA.

Upon consideration of the gender of individuals with absent 39 kD HLA, it was found that all 7 of the HLA-B7 individuals lacking the 39 kD HLA were female (Table 4). Furthermore, the association between female gender and absence of 39 kD plasma HLA, disregarding B7 allele, was significant ($p=0.03$). The only exception to HLA-B7 allele and female gender in individuals with absent 39 kD HLA was a male who is homozygous for HLA-A1 and -B8. These results suggest that HLA-B7 allele and female gender may negatively influence alternative splicing in the generation of water soluble 39 kD plasma HLA.

Table 3. Frequency of HLA-A or -B Phenotypes Among Individuals Included in the Study.

HLA-A	No.(%)	AF(%)[a]	HLA-B	No.(%)	AF(%)[a]
1	10(22.7)	25.7	5	2(4.5)	9.3
2	19(43.2)	46.6	7	12(27.2)	18.7
3	13(29.5)	26.0	8	11(25.0)	17.1
9	1(2.3)	5.0	13	1(2.3)	5.3
11	8(18.2)	12.5	14	3(6.8)	9.5
23	1(2.3)	5.0	18	1(2.3)	9.7
24	10(22.7)	12.8	22	6(13.6)	5.4
25	4(9.1)	4.2	27	4(9.1)	7.5
26	3(6.8)	7.2	35	3(6.8)	15.6
28	2(4.5)	9.9	37	2(4.5)	3.2
29	3(6.8)	8.1	39	3(6.8)	3.6
30	1(2.3)	5.1	40	4(9.1)	13.0
			44	6(13.6)	26.1
			45	1(2.3)	1.4
			50	1(2.3)	2.6
			51	1(2.3)	9.3
			53	1(2.3)	0.9
			57	1(2.3)	7.2
			60	5(11.4)	11.0
			62	9(20.4)	9.5

Total: 44 (21 Male, 23 Female)

[a]AF is the published antigen frequency for American Caucasians (48).

Figure 7.Association of HLA phenotype with undetectable 39 kD plasma HLA antigens. Of individuals lacking 39 kD plasma HLA antigens (n=8), 87.5% are HLA-B7 positive. The association between HLA-B7 allele and absent 39 kD plasma HLA is statistically significant as 7 of 12 HLA-B7 positive individuals lacked the 39 kD plasma HLA while only 1 of 32 HLA-B7 negative individuals did not express the 39 kD plasma HLA ($p<0.00015$ by Fisher's Exact Test). The 50% association seen with HLA-A3 may be explained by linkage disequilibrium between HLA-A3 and HLA-B7 (47).

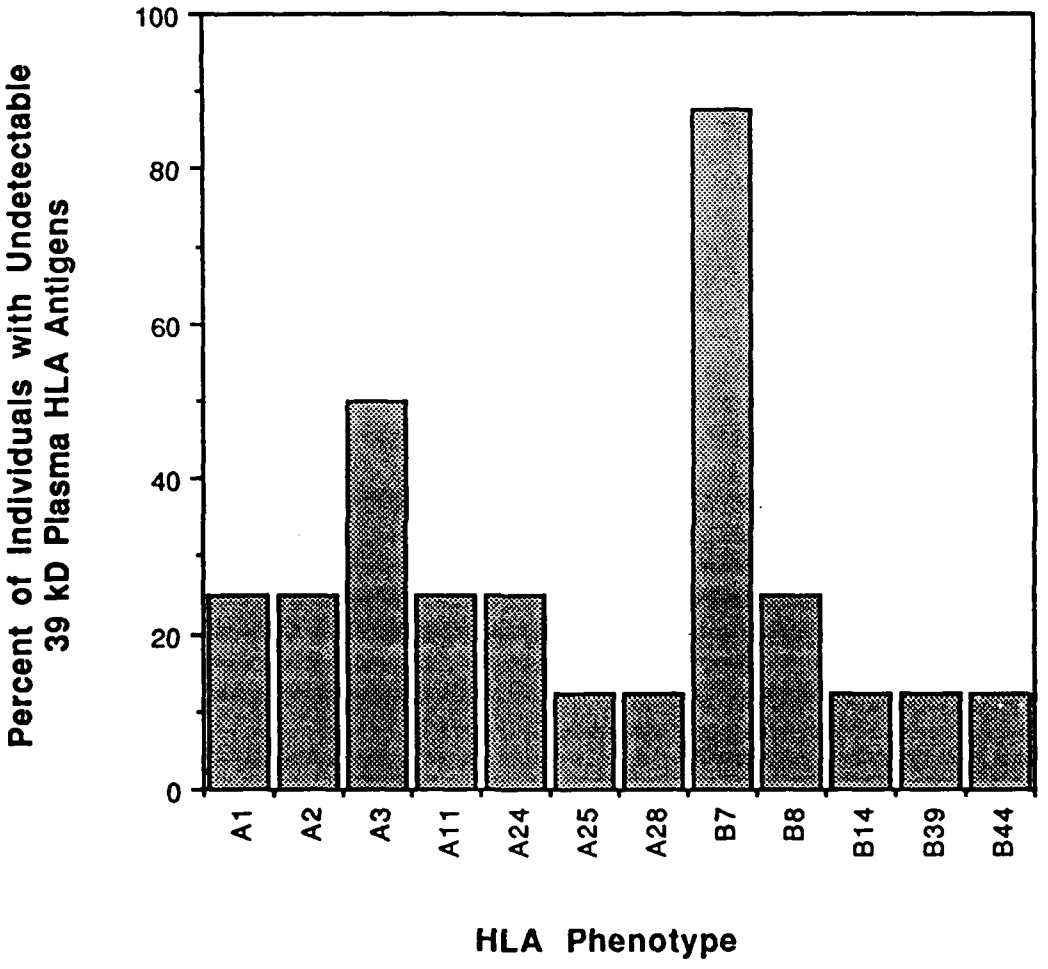

Percent of Individuals with Undetectable 39 kD Plasma HLA Antigens
100
80
60
40
20
0
A1
A2
A3
A11
A24
A25
A28
B7
B8
B14
B39
B44
HLA Phenotype

Table 4. HLA Phenotypes and Gender of Individuals With Absence of 39 kD Plasma HLA Antigens.

			HLA Phenotype			
No.	Ind.[a]	Sex	A		B	
1	DM	M	1	1	8	8
2	AB	F	2	3	7	-[d]
3	EB	F	3	24	7	-
4	BS	F	1	3	7	8
5	KK	F	3	24	7	39
6	DR[b]	F	11	28	7	14
7	ER[b]	F	11	25	7	44
8	KV[c]	F	2	2	7	7
9	EH[c]	F	1	2	7	8
10	AA[c]	F	1	2	7	8

[a]Individuals 1-8 were identified in the population study. Individuals 9 and 10 were subsequently identified during pedigree analysis of families of HLA-B7 positive and 39 kD negative probands.
[b]Mother (ER) and daughter (DR).
[c]Mother (EH) and daughters (KV and AA).
[d]- = unknown antigen or blank.

Pedigree Analysis of Four Families of HLA-B7 Positive and 39 kD Negative Probands

In order to establish the direct association between absence of 39 kD plasma HLA and B7 allele and female gender, pedigree analysis of four families of HLA-B7 positive, 39 kD negative probands was performed. Samples were collected from each family member for HLA phenotyping and evaluation of patterns of plasma HLA as described previously. The results are shown in Figure 8.

In family 1, both the mother (ER) and daughter (DR) share a haplotype with HLA-B7 allele and do not express 39 kD plasma HLA. No other family members are HLA-B7, nor do they lack the 39 kD plasma HLA. It is of interest to note that the presence of both 36 and 34 kD HLA in the plasma of the father (AR) and one daughter (DR), is associated with the A28, B14 haplotype. This correlates with the finding of Dobbe et al. that HLA-A28 appears to associate with the presence of both 35 and 37 kD HLA in the plasma (28).

Although the results from family 1 suggest only that absence of 39 kD plasma HLA may be associated with HLA-B7, subsequent evaluation of three additional families of probands with absence of 39 kD plasma HLA further indicates that HLA-B7 allele and female gender are necessary but not sufficient to inhibit the generation of class I HLA through alternative splicing of mRNA.

In family 2, the HLA-B7 positive, 39 kD negative female proband (KV) is homozygous HLA-A2, -B7. The mother (EH), the proband (KV), and the other daughter (AA) are all HLA-B7 and lack the 39 kD plasma HLA. Although the father (AH) does have the HLA-B7 allele, he does not lack

the 39 kD plasma HLA. Additionally, the B7 allele in daughter (AA) is inherited from the father (AH). This finding suggests the additional requirement of female gender for the absence of 39 kD plasma HLA.

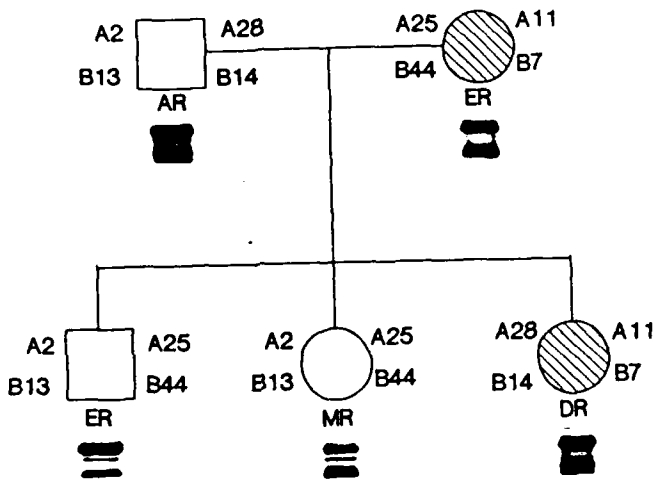
In family 3, the B7 allele is found in the father (WK) and two daughters (DK) and (KK). Only one daughter, (KK) demonstrates the absence of detectable 39 kD plasma HLA. While reemphasizing the importance of B7 allele and female gender, this finding also indicates that B7 allele and female gender are not the sole factors influencing absence of 39 kD HLA in plasma.

In family 4, the mother (ES), two male siblings (RS and BS) and the female proband (BS) are all HLA-B7 positive. However, only the proband (BS) lacks the 39 kD plasma HLA. The results from family 4 also suggest that genetic factor(s) not associated with HLA-B7 allele and female gender are required to inhibit alternative splicing of class I HLA mRNA.

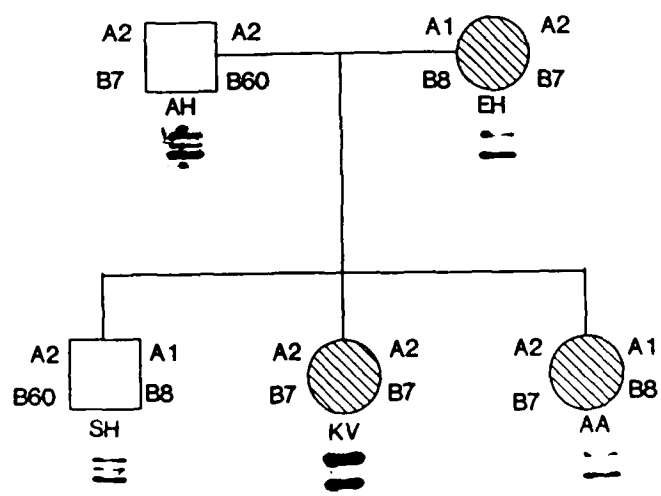
Figure 8. Pedigree analysis of four families of HLA-B7 positive and 39 kD negative probands. Plasma samples were collected and frozen at -70°C until immunoprecipitated with W6/32 anti-HLA MoAb and analyzed by SDS-PAGE and immunoblot with HC-10 anti-HLA HC MoAb. Open squares and open circles represent males and females, respectively, who express 39 kD plasma HLA. Hatched circles are females with absence of 39 kD plasma HLA. Individual plasma HLA patterns appear below the initials. Families 1 and 2 are healthy volunteers; families 3 and 4 are diabetic families (at least one family member has type I diabetes) followed at the Clinical Research Center at the University of Florida.

Family 1: Proband is DR
Family 2: Proband is KV
Family 3: Proband is KK
Family 4: Proband is BS (female)

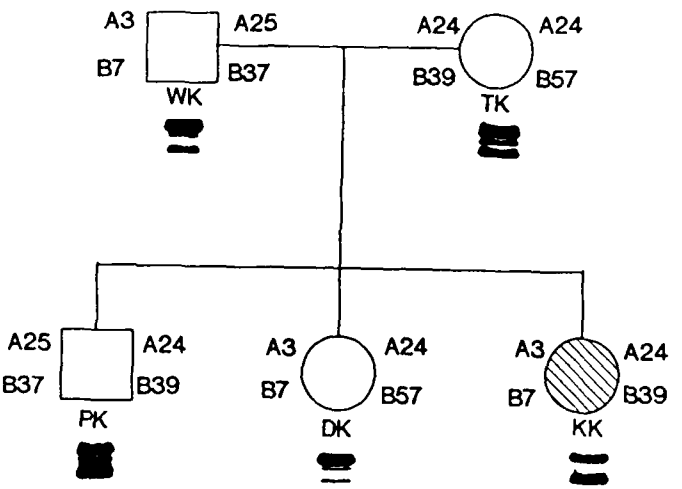
FAMILY 1



FAMILY 2



FAMILY 3



FAMILY 4

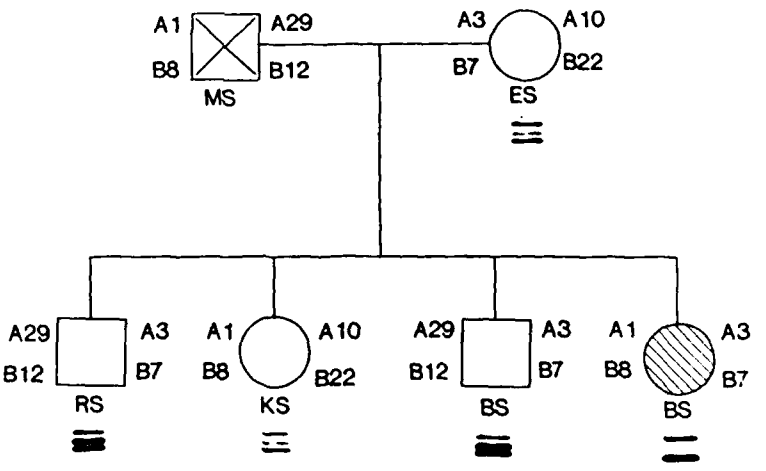


Figure 8--continued.

DISCUSSION

Although the presence of different molecular weight forms of HLA antigens in plasma was described previously, reports vary with regard to the number of different forms. Krangel (27) reported two forms of HLA in plasma, an intact, lipid-associated 43 kD HLA and a water-soluble 39 kD HLA, while Dobbe et al. (28) detected water-soluble HLA from 35 to 37 kD in addition to 44 and 40 kD plasma HLA. Moreover, whether the distribution of different molecular forms of HLA antigens in the plasma of different individuals are the same or not has not been reported.

Regarding the origin of HLA antigens in plasma, it is generally agreed that the intact 44 kD HLA are generated by membrane shedding of cellular HLA antigens (21, 23, 27, 28). Two different mechanisms, however, have been proposed to explain the presence of 39-40 kD HLA antigens in plasma. The first mechanism, described by Krangel (27), is that alternative splicing of class I HLA messenger RNA excises exon 5 which encodes the transmembrane domain of HLA antigens. This produces a molecule that does not have the hydrophobic transmembrane domain, and therefore, is secreted from the cell rather than bound to the lipid-bilayer of the cell membrane. Alternately, as suggested by Dobbe et al., 39-40 kD plasma HLA as well as 35-37 kD plasma HLA may be equivalent to Q10 molecules in mice. Q10 molecules are non-polymorphic class I MHC molecules with an altered transmembrane domain and deletion of the cytoplasmic tail. They are synthesized in the liver and secreted

in the plasma (49, 50). Q10 antigens are products of an additional class I locus (Qa) in the mouse (50), for which a human counterpart has not been identified. In addition, Dobbe and co-workers (28) suggested that the lower molecular weight forms of HLA antigens in plasma, specifically the 35-37 kD HLA, may also be products of proteolytic degradation of intact HLA antigens.

Evidence which suggests that alternative splicing of mRNA is the mechanism responsible for generation of 39-40 kD plasma HLA includes the finding that alternative splicing, with deletion of the transmembrane domain, occurs in a mutant B lymphoblastoid cell line (25) and like the alternatively-spliced HLA antigens from this cell line, plasma HLA of similar molecular weight is not extractable by TX-114 (27).

Evidence which supports that lower molecular weight forms of plasma HLA are generated by proteolytic degradation of intact 44 kD HLA was provided by Dobbe and co-workers (28). Their results indicated that a regular cytoplasmic tail is not present in most of the 40 kD plasma HLA or in any of the 35-37 kD plasma HLA evaluated in their study (28). Nevertheless, their results did not exclude the possibility that the antibody failed to react with the cytoplasmic tail due to alterations in its sequence or structure as a result of deletion of the transmembrane domain.

The intent of this study was to 1) identify the different molecular weight forms of HLA in plasma, 2) determine the distribution of the different molecular weight forms of plasma HLA in the general population, 3) characterize plasma HLA biochemically in order to gain information regarding the genesis of soluble forms of HLA antigens, and

4) determine whether there is any association between HLA phenotype and presence of the different molecular weight forms of plasma HLA.

In contrast to earlier findings that demonstrated a single plasma peak containing HLA activity at an apparent molecular weight of 190 kD (23), we identified two plasma pools containing HLA antigen by gel filtration chromatography. Pool I had an apparent molecular weight of 200 kD and contained intact 44 kD HLA antigen, while pool II appeared at a molecular weight of approximately 50 kD and contained 39 and 36 kD HLA. Since W6/32 anti-HLA MoAb, which recognizes a conformational epitope dependent on the association of HLA heavy chain with β_2m, was used to immunoprecipitate plasma HLA, the different molecular weight forms of plasma HLA heavy chains detected on immunoblot with HC-10 anti-HLA heavy chain MoAb most likely were associated with β_2m. As suggested by Aster et al. (21) and Allison et al. (23), intact 44 kD HLA in plasma is probably lipid-associated and results in the high molecular weight observed for 44 kD HLA in plasma by gel filtration chromatography. Since gel filtration chromatography in the earlier study (23) was performed on the HDL fraction of serum, only lipid-associated 45 kD HLA antigen was detected. Conversely, the second pool of plasma HLA has an apparent molecular weight of 50 kD which approximates the molecular weight of 12 kD β2m in complex with 36 and 39 kD HLA heavy chains. Thus, the results suggest that the second pool of plasma HLA represents a water soluble form of the protein complex without any lipid assocation. The hydrophobic transmembrane domain, therefore, may not be present in the 39 and 36 kD HLA. This interpretation, indeed, was substantiated by the subsequent TX-114 extraction study which

demonstrated that only 44 kD plasma HLA is hydrophobic and extractable by TX-114.

In order to demonstrate whether the lower molecular weight forms of HLA antigens with the loss of the transmembrane domain could be derived from degradation of intact 44 kD HLA antigens in blood, *in vitro* degradation of HLA antigens in plasma and whole blood was studied. No change in the relative quantity of each molecular weight form of HLA in plasma and whole blood was observed after incubating *in vitro* for more than 48 hours. When the blood from an individual with absent 39 kD plasma HLA was studied, there was no detectable 39 kD HLA in plasma even after incubation for 72 hours. Nor did 34 kD HLA antigens (subsequently demonstrated in the population study) appear in any of the plasmas or whole blood samples evaluated (none contained 34 kD HLA prior to incubation). These results suggest that degradation of intact 44 kD HLA antigens in blood is unlikely to be responsible for generating the lower molecular weight forms of plasma HLA. This does not rule out, however, an intracellular process which degrades or cleaves HLA proteins prior to release from cells.

Whether the lower molecular weight forms of plasma HLA are derived by alternative splicing with deletion of the transmembrane domain or proteolytic cleavage of the cytoplasmic tail and transmembrane domain from intact 44 kD HLA, the NH_2-terminal amino acid sequence of each form of plasma HLA should be identical. With the exception of the NH_2-terminal amino acid in three HLA-C alleles, the first 5 to 6 amino acids in 39 HLA-A, -B and -C alleles sequenced thus far are identical (15). Different sequences would be obtained if proteolysis resulted in

cleavage of the NH_2-terminus from intact 44 kD HLA antigens or if the NH_2-terminus was changed due to a shift in the DNA reading frame as a result of alternative splicing or use of an alternate splice acceptor site. A different NH_2-terminal sequence could also be due to the presence of class I molecules encoded by *class I MHC genes other than* HLA-A, -B or -C, or to the presence of a completely unrelated protein. Analysis of the NH_2-terminal amino acid sequence of the 44, 39 and 36 kD HLA antigens in plasma revealed that the 44 kD and 39 kD molecules had the same sequence as intact cellular HLA antigens. This result suggests that the 39 kD HLA in plasma does not arise by proteolytic cleavage of the NH_2-terminus from intact 44 kD HLA and further supports that 39 kD plasma HLA, with loss of the transmembrane domain, may be generated by alternative splicing. Unexpectedly, the NH_2-terminal sequence of the 36 kD plasma HLA was found to be unique. This result suggests that 36 kD plasma HLA is not generated solely by proteolytic removal of the cytoplasmic tail and transmembrane domain from intact 44 kD HLA-A, -B, or -C antigens as suggested previously (28). The 36 kD molecules in plasma may then be 1) a product of the classical class I loci, lacking the transmembrane domain and having an altered NH_2-terminus, 2) a novel class I antigen, or 3) an unrelated protein.

Direct comparison of plasma HLA antigens and papain-digested cellular HLA separated simultaneously on SDS-polyacrylamide gel demonstrated that 39 kD plasma HLA has the same molecular weight as the first proteolytic product obtained from papain digestion of purified cellular HLA antigens. As reported previously (16,43,44), the 39 kD papain-digested HLA has lost the cytoplasmic tail, but retains the

intact transmembrane domain and is extractable by TX-114. In contrast, the 39 kD plasma HLA is not extractable by TX-114 and may not contain the hydrophobic transmembrane domain. The lack of extractability by TX-114 also precludes the possibility that 39 kD plasma HLA represents nonglycosylated intact HLA. The nonglycosylated intact HLA with presence of the hydrophobic transmembrane domain should be extractable by TX-114. Since the molecular weight of 39 kD plasma HLA includes an intact NH_2-terminus and it is too large to be explained by the loss of the cytoplasmic tail and transmembrane domain, the only logical explanation would be that 39 kD plasma HLA is produced through alternative splicing with deletion of the transmembrane domain as suggested previously (27).

In order to acquire direct biochemical evidence to support that 39 kD plasma HLA is generated through deletion of the transmembrane domain, attempts were made to compare the carboxyl-terminus of 44 kD HLA antigens and 39 kD plasma HLA by development of a monoclonal antibody against a conserved amino acid sequence in the cytoplasmic tail and also by carboxypeptidase Y digestion of purified HLA antigens without any success. Nevertheless, the results as discussed above support the earlier suggestion made by Krangel (27) that 39 kD plasma HLA antigens are generated by alternative splicing of messenger RNA, resulting in deletion of the transmembrane domain.

Recently, non-classical class I MHC molecules were reported and were mapped telomeric to the HLA-A locus (4, 51, 52). The nonclassical or class I-like antigens generally differ from the classical HLA-A, -B, and -C antigens in that they are relatively nonpolymorphic, they have a

restricted tissue distribution, and they do not share epitopes with the classical class I HLA antigens (51). Antigens expressed on activated T lymphocytes, such as the Human T locus or HT system proposed by Gazit (reviewed in references 51 and 52), and the Human Activated (HA) system proposed by Fauchet et al. (53), as well as the TCA and TCB diallelic systems identified on subsets of T lymphocytes by van Leeuwen et al. (54, 55) are included in this group of class I-like molecules encoded by genes linked to the HLA-A locus. They generally consist of a heavy chain of reduced molecular weight as compared to HLA antigens, ranging approximately 40-42 kD, and are associated with β_2m (51-55). However, as the T cell antigens described do not possess the monomorphic epitope recognized by W6/32 anti-HLA MoAb (51-55) that is present on plasma HLA antigens as well as on cellular HLA antigens, the lower molecular weight forms of plasma HLA therefore do not correspond to T cell antigens.

More recently, Shimizu et al. (56) identified three new class I HLA molecules encoded by class I genes different from HLA-A, -B and -C genes. The 6.2 or recently named HLA-E gene (57) is located between the HLA-A and -C genes and encodes a 41 kD protein that associates with β_2m. Although it encodes an intact transmembrane domain, it does not appear to be expressed on the cell surface and is detected intracellularly (56, 57). It was suggested that this molecule might be secreted, similar to the Qa antigens in the mouse (57). Although the HLA-E antigen is precipitated by W6/32 and is similar in molecular weight to the soluble 39 kD HLA detected in plasma, the presence of an intact transmembrane domain suggests that this molecule would be extractable by TX-114 and is unlike 39 kD plasma HLA. Shimizu et al. (56) and Geraghty et al. (58)

also identified new HLA antigens of 38 and 40 kD associated with β_2m. Again, the 38 kD HLA contains an intact transmembrane domain and is expressed at the cell surface (56, 58). Therefore, it is not likely to be the 39 kD plasma HLA. The 40 kD HLA, like the 41 kD HLA-E antigen is not expressed at the cell surface (56). As its sequence has not been published and available data does not characterize the transmembrane domain, it cannot be evaluated with regard to the 39 kD plasma HLA.

Although direct evidence demonstrates that alternative splicing of class I HLA mRNA occurs in cell lines to produce a secretable form of HLA antigen, this same evidence is not available for normal cells. However, 39 kD plasma HLA has been characterized and appears to be identical to alternatively-spliced antigens detected in cell lines. Further evidence, including characterization of the cytoplasmic tail of plasma HLA antigens or detection of alternatively-spliced mRNA in normal cells, would definitively identify 39 kD plasma HLA as an alternatively-spliced, secretable HLA antigen.

As to the 36 kD plasma HLA, its biochemical and immunological relationship to classical class I HLA and its genesis remain to be further investigated. At present, 36 kD plasma HLA can be considered a class I molecule based on the presence of epitopes recognized by both W6/32 anti-HLA MoAb and HC-10 anti-HLA heavy chain MoAb. Furthermore, it does not possess a transmembrane domain and has a unique NH_2-terminus. These findings, as well as <u>in vitro</u> degradation studies, suggest that the 36 kD plasma HLA is not generated by proteolytic degradation of 39 or 44 kD HLA antigens.

The characterization of 34 kD plasma HLA demonstrated in the population study discussed below was not performed due to the limited quantity of plasma samples.

After studying plasma HLA in 44 HLA-phenotyped individuals, four different molecular weight forms of plasma HLA were identified. The detection of four different molecular weight forms of HLA antigens in plasma correlates with the finding of Dobbe et al. (28). Although similar methodology was used by Krangel (27), only 43 and 39 kD HLA was identified in plasma in his study. An explanation for his failing to observe 36 and 34 kD plasma HLA is not readily apparent; however, the difference in anti-HLA heavy chain antibodies used for immunoblot may be responsible for the discrepancy.

The results of our studies also indicated that there are four different plasma HLA patterns in the general population. Since quantitative expression of plasma HLA was reported to be influenced by HLA phenotype (22,23,27-29), the possible association of HLA phenotype with the four different patterns of plasma HLA antigens identified in our population was investigated. The only association between HLA phenotype and different molecular weight forms of plasma HLA was between HLA-B7 allele and absent 39 kD plasma HLA antigen. Seven of 8 individuals with absent 39 kD plasma HLA antigen were positive for HLA-B7 allele. Furthermore, all 7 of these individuals were female. The only exception was a male homozygous for HLA-A1 and -B8. These results suggested that HLA-B7 allele and female gender may inhibit the generation of 39 kD plasma HLA antigens by alternative splicing.

Pedigree analysis of four families of HLA-B7 positive and 39 kD plasma HLA negative probands (all female), further supports that B7 allele and female gender are necessary but insufficient for absence of 39 kD HLA in plasma. The requirement of female gender became evident when a daughter with absence of 39 kD plasma HLA inherited B7 phenotype from the father with presence of 39 kD plasma HLA antigens. The finding that one of the probands inherited the B7 gene from her mother who did express 39 kD HLA in plasma suggested that genetic factors other than B7 gene and female gender were necessary to result in absence of 39 kD HLA in plasma. Therefore, HLA-B7 allele, female gender, and other genetic factor(s) may determine the inhibition of alternative splicing of HLA antigens and result in absence of 39 kD HLA antigens in plasma.

Since all of the plasma HLA samples included in the population study and the pedigree analyses were evaluated on immunoblot with HC-10 anti-HLA heavy chain MoAb, the results must be evaluated in light of the specificity of HC-10. HC-10 has been shown to have preferential reactivity to HLA-B locus heavy chains (31). Therefore, plasma HLA detected by immunoblot represents HLA-B antigens primarily and any interpretation with regard to HLA-A antigens must be made with caution. More specifically, the absence of 39 kD HLA antigens may actually reflect an absence of HLA-B antigens with inadequate detection of HLA-A antigens. However, as noted by Stam, et al., HC-10 reacts very well with HLA-A3, and 50% of individuals lacking the 39 kD plasma HLA are HLA-A3 as well as HLA-B7. Therefore, factors resulting in inhibition of alternative splicing may affect HLA-A antigens as well as HLA-B

antigens. The exact mechanism defining the association of HLA-B7 gene and female gender with the possible inhibition of alternative splicing of HLA genes remains to be elucidated.

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BIOGRAPHICAL SKETCH

Jo Ann Haga graduated from Louisiana Technical University, Ruston, Louisiana, in 1979 with a Bachelor of Science degree in medical technology and was subsequently certified by examination as a Medical Technologist by the American Society of Clinical Pathologists and as a Clinical Laboratory Scientist by the American Society of Medical Technologists. While at Louisiana Technical University she entered the Air Force Reserve Officer Training Corps and was commissioned as a second lieutenant in the United States Air Force in August of 1979. After serving on active duty for seven years as a Biomedical Laboratory Officer, she was accepted into the Air Force Institute of Technology, Civilian Institute program to earn a Master of Science degree in clinical chemistry. She began graduate studies at the University of Florida in the Department of Pathology and Laboratory Medicine in the fall of 1987 and completed a Master of Science degree in medical sciences. She was then assigned as the Associate Chief of Clinical Chemistry at Wilford Hall Medical Center, Lackland Air Force Base, San Antonio, Texas, where she plans to continue her career as a clinical chemist and officer in the United States Air Force.

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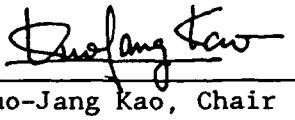

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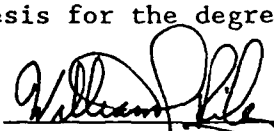

William J. Riley
Associate Professor of Pathology
and Laboratory Medicine

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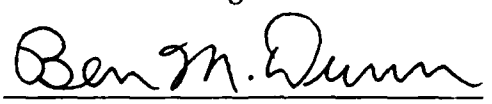

Ben M. Dunn
Professor of Biochemistry and
Molecular Biology

This thesis was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Master of Science.

December 1989

Dean, College of Medicine

Dean, Graduate School